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Controlled Release from Cleavable Polymerized Liposomes upon Redox and pH Stimulation

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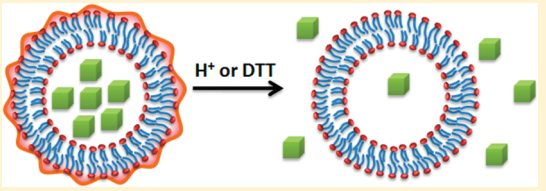
Supporting Information

ABSTRACT: A gallate derivative with three propargyl groups was coupled to palmitoyl oleoyl phosphoethanolamine (POPE). The resulting anionic lipid was formulated with common lipids such as palmitoyl oleoyl phosphatidyl choline (POPC) to form large unilamellar vesicles (LUVs). Polymerization of the LUVs was accomplished by the Cu(I)-catalyzed click reaction between the propargyl groups and the azide groups in the cross-linker. When the cross-linker contained a disulfide or ketal group, the resulting polymerized liposomes depolymerized and released entrapped contents upon the addition of a reducing thiol or under weakly acidic conditions. The click reaction allowed simultaneous multivalent surface functionalization during cross-linking, making these cleavable polymerized liposomes (CPLs) potentially very useful in the delivery and controlled release of pharmaceutical agents.



An ideal drug-delivery vehicle unloads its contents only at the target site. Liposomes have many of the desired features of "smart" drug-delivery systems including large encapsulating capacity, ease of surface decoration, and biocompatibility.[1−7] A number of liposomal drug formulations have been approved by FDA and numerous others are undergoing clinical trials.[1−7] In recent years, there has been growing interest in developing liposomes that release their contents under specific stimuli including pH,[5,8−19] enzymes,[20−23] temperature,[24] redox signals,[25−27] and light.[12,28−32] The majority of the reported methods focus on the design of responsive lipids. Typically, the stimulus triggers changes in the lipid structure and, in turn, causes destabilization and leakage of liposomes.

An important challenge faced by liposomal delivery lies in the stability of the noncovalently assembled structures. Formation of liposomes is driven by hydrophobic interactions among the lipid tails. After entering the vascular system, liposomes are destabilized through the loss of lipid molecules to hydrophobic entities such as biomembranes and plasma proteins, resulting in premature leakage of entrapped contents. Many approaches have been developed to improve liposomal stability, including tuning of membrane fluidity, incorporation of cholesterol, and surface protection with poly(ethylene glycol).[1−7] More recently, cholesterol-derived lipids[33,34] and polymers[35] were found to be particularly effective at enhancing liposomal stability.

Polymerization of liposomes, whether near the headgroup or in the lipid tails, can stabilize the structure as well.[36−40] Although some polymerized liposomes are known to survive harsh treatment including dehydration and rehydration, their usage in clinical drug delivery is hampered by the poor biocompatibility of the polymerized materials. Whereas most lipids used in common liposomal formulations are bio-originated and thus fully biodegradable, the polymerized materials, often connected by carbon−carbon bonds, are macromolecules very difficult to degrade.

In this paper, we report a simple method to prepare cleavable polymerized liposomes (CPLs). Our approach combines the simplicity of polymerization-derived stabilization with the flexibility of small-molecule lipids. Our method can be easily applied to common lipid formulations to create liposomes with controlled-release properties under biologically relevant conditions. The chemistry employed to polymerize the liposomes permits facile conjugation of the liposomes with functional ligands such as fluorescent labels, making it convenient to track the fate of the liposomes.

Polymerizable lipid **1** is a derivative of common POPE lipid. As shown by Scheme 1, a propargylated gallic acid (**2**) was activated by *N*-hydroxysuccinimide (HOSu). The resulting activated ester (**3**) reacted with the amino group of POPE in the presence of diisopropylethylamine to afford **1**. The product was purified by column chromatography and characterized by ^{1}H and ^{13}C NMR spectroscopy, as well as mass spectrometry. The three propargyl groups of **1** are included for polymerizing the liposomes by azide-containing cross-linkers such as **4** and **5** through the highly efficient click reaction (i.e., the 1,3-dipolar cycloaddition between terminal alkyne and azide). The high-yield, selectivity, and outstanding tolerance of functional groups of the click reaction[41−43] enabled its numerous applications in biolabeling,[44,45] surface modification,[46] dendrimer synthesis,[47−49] and polymer formation.[46] Click chemistry has also been applied to liposomes in recent years,[35,50−55] but mostly for their conjugation with ligands or functional labels.

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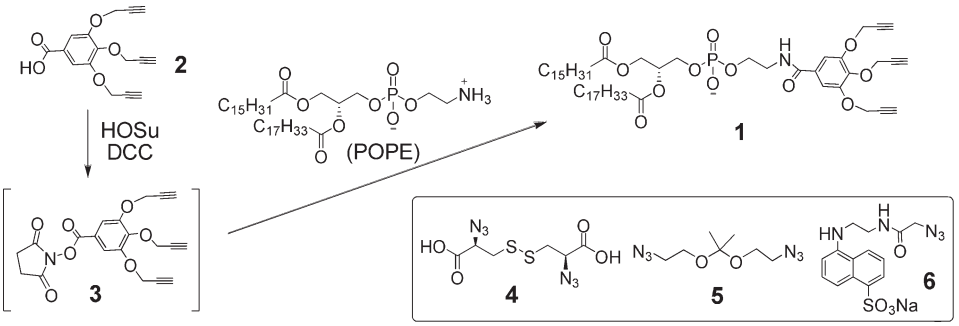
Scheme 1. Synthesis of Cross-Linkable Lipid 1



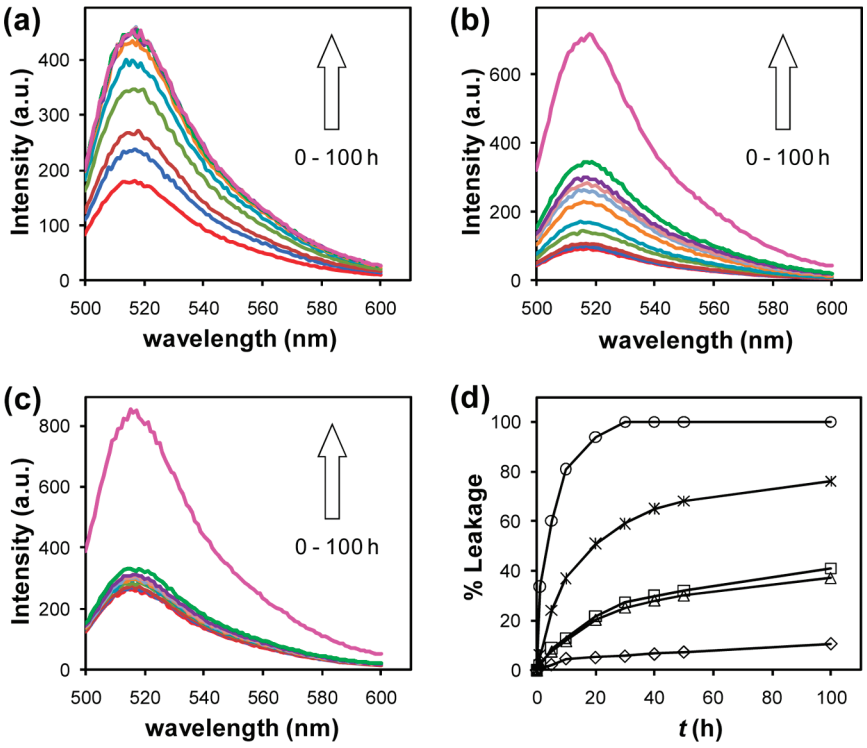


Figure 1. Fluorescence spectra of CF-containing LUVs prepared with (a) **1**, (b) 1:1 [**1**]/[POPC], and (c) POPC at ambient temperature. $[CF]_{initial}$ = 50 mM in 10 mM HEPES buffer. [NaCl] = 0.1 M. The spectra were recorded at 0, 0.5, 1, 5, 10, 20, 30, 40, 50, and 100 h after the liposomes were prepared. (d) Percent leakage of CF from LUVs prepared with different lipid formulations. [**1**]/[POPC] = 1:0 (○), 2:1 (×), 1:1 (□), 1:2 (△), 0:1 (◇).

Lipid **1** could be easily formulated with POPC to form liposomes. We employed the well-established carboxyfluorescein (CF) leakage assay[56] to understand the effect of lipid formulation on liposomal stability. Briefly, CF was trapped inside large unilamellar vesicles (LUVs) at a concentration (50 mM) that gave significant self-quenching. Any leakage of the dye would dilute the probe and enhance its fluorescence. Figure 1a−c shows the fluorescence spectra of several CF-containing LUVs over a period of 100 h. The spectra in violet were recorded at the end of the 100 h after 1% Titron X-100 aqueous solution was added to lyse the liposomes. The spectrum after Triton addition thus corresponded to 100% leakage and the percent leakage of CF at different times was calculated accordingly and summarized in Figure 1d.[57] From the leakage profile, we can easily see that, by itself, **1** did not make very stable liposomes—the majority of CF leaked out in 10−20 h (Figure 1d, ○). The stability of the liposomes improved significantly when **1** was formulated with POPC. The liposomes with **1**/POPC = 1:1 or 1:2, for example, retained over 60% of the dye at 100 h.

The POPC LUVs leaked very little even at 100 h (Figure 1c). Because polymerized liposomes are expected to be more stable than unpolymerized ones, the normal CF assay might not be sensitive enough for their characterization. A common way to speed up the leakage is to apply an osmotic gradient across the membranes.[33,58−60] Indeed, the LUVs with [**1**]/[POPC] = 1:1 lost all the entrapped CF in 5 M NaCl in only 12 h (Figure 2a, ▲), whereas the same lost <40% after 100 h when the buffer contained 0.1 M NaCl (Figure 1d, □). An increase of POPC in the formulation stabilized the liposomes (Figure 2a), as it did without the osmotic stress. Note that, in all the assays, the leakage

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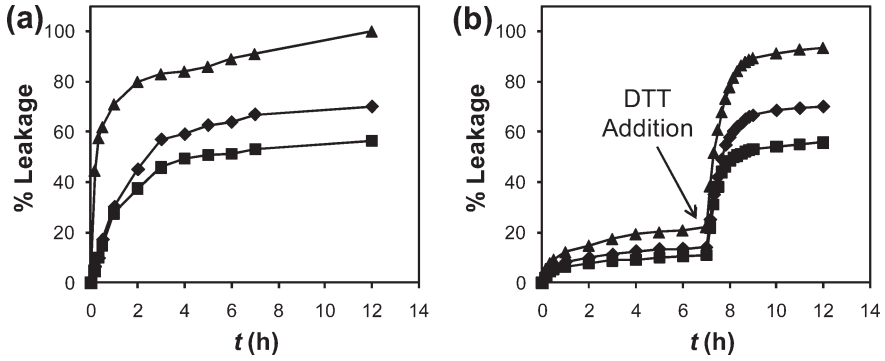

Figure 2. Percent leakage of CF from LUVs (a) before and (b) after polymerization. [CF] = 50 mM in 10 mM HEPES buffer. [NaCl] = 5 M; [**1**]/[POPC] = 1:1 (▲), 1:2 (♦), and 1:5 (■); DTT = dithiothreitol.

was fast initially and leveled off afterward. This was a reasonable result. The exchange of water and/or salt probably occurred when the dye escaped from the liposomes. As the osmotic pressure decreased across the lipid membranes, the driving force for leakage became smaller over time.

Polymerization of the LUVs was accomplished by $CuCl_2$/sodium ascorbate in the presence of 1.5 equiv of cross-linker **4**.[61] The stoichiometry was chosen because **1** contained three alkynyl groups and **4** two azides. The organic HEPES buffer was found to interfere with the click reaction and a white precipitate formed when Cu(II) salt was added to the buffer. Assuming the precipitate was a complex between copper ions and the organic amine HEPES, we added 4 equiv of *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA), a water-soluble amine known to complex with the copper catalyst used in the click reaction.[62] The click reaction indeed proceeded smoothly (vide infra) without any precipitation. The experimental details are given in the Supporting Information.

We polymerized liposomes with three different formulations —[**1**]/[POPC] = 1:1, 1:2, and 1:5. Liposomes with higher POPC are more stable according to Figure 1d but would give a lower degree of polymerization. As expected, polymerization suppressed the CF leakage to a large extent in all three liposomes. Instead of 50−90% leakage prior to polymerization (Figure 2a), the cross-linked LUVs displayed 10−20% leakage when placed in 5 M NaCl solution before dithiothreitol or DTT was added (Figure 2b). It was surprising, however, that the relative stability of the three liposomes (■ > ♦ > ▲) stayed the same, and the most polymerized liposomes (▲) were the least stable among the three. Polymerization clearly improved the liposomal stability, but it was puzzling that the degree of polymerization did not correlate with the stability. Possibly, the sensitivity toward osmotic pressure derived partly from the ionic nature of lipid **1** (which would not change upon polymerization). Figure 1d indicates that neutral POPC is far more resistant to leakage than the anionic **1**. Regardless of the exact reason for the retained order of stability, the result was actually quite desirable. For any practical applications, one would rather not have to use a lot of a specially synthesized lipid to improve stability.

The disulfide-containing cross-linker (**4**) permitted the cleavage of the polymerized liposomes with a commonly used reducing thiol, DTT. Because thiol concentration reaches 10 mM in the cytosol and is only about 10 μM in the plasma, the disulfide−thiol conversion is widely used for cellular delivery.[63] Thiol-triggered release is also useful for delivering anticancer drugs because the thiol concentration could be seven times higher near some tumors than in normal tissues.[64]

To our gratification, DTT (ca. 0.3 mM) was able to completely reverse the effect of polymerization on the CF leakage. Fast leakage started as soon as the thiol was added and the leakage profiles were nearly identical to those before the polymerization (compare Figures 2a and 2b after DTT addition). In our method, the copper catalyst was added after the formation of the liposomes and the water-soluble copper catalyst should not permeate the bilayers easily. Polymerization, as a result, was anticipated to occur predominantly on the external leaflets of the bilayers. Such a configuration is beneficial from the viewpoint of release, as it is certainly easier to cleave the disulfide bonds in the external leaflets than those in the interior by external stimuli.

For the polymerized LUVs, mass spectrometry indeed revealed high M.W. species (Figure 1S, Supporting Information). Nonetheless, since there was no guarantee that all polymeric species ionized to the same extent, the peaks in the MS might not accurately represent the polymerized lipids. To better understand the click polymerization, we took advantage of the reversible nature of the cross-linkage and studied the depolymerized LUVs to probe the cross-linking density. This method was used previously to characterize similar, click-cross-linked materials.[61] The depolymerized LUVs were obtained after the CPLs ([**1**]/[POPC] = 1:1) were incubated with 0.3 mM DTT for 24 h and were then immediately analyzed by ESI-MS. Although we could not assign every single peak due to the complexity of the system, two major peaks in the mass spectrum were identified as **7** (m/z = 1229.59) and **8** (m/z = 1276.60). We did not detect any significant amount of **9** (m/z = 1424.62). Thus, the MS results indicated that the majority of lipid **1** underwent two cycloadditions and some reacted only once under our experimental conditions.

There could be several reasons why the click reaction did not result in high cross-linking density. The click reaction was performed in fairly dilute liposomal solutions, with [**1**] = 0.26 mM. Since both the liposomes and the cross-linked **4** were anionic, it could be difficult for the cross-linker to access the alkynyl groups of the anionic **1**. Moreover, because of the hydrophobicity of the propargylated gallate, the alkynyl groups could be buried within the hydrophobic membranes instead of being exposed to the aqueous phase. Finally, electrostatic interactions among the lipids demanded that the anionic **1** be dispersed as far from one another as possible in the matrix of POPC. All of these could have contributed to the low cross-linking density of the CPLs. It should be noted, nevertheless, that

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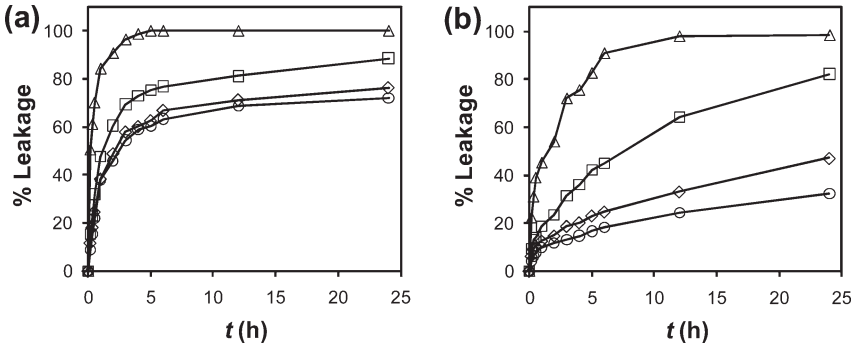
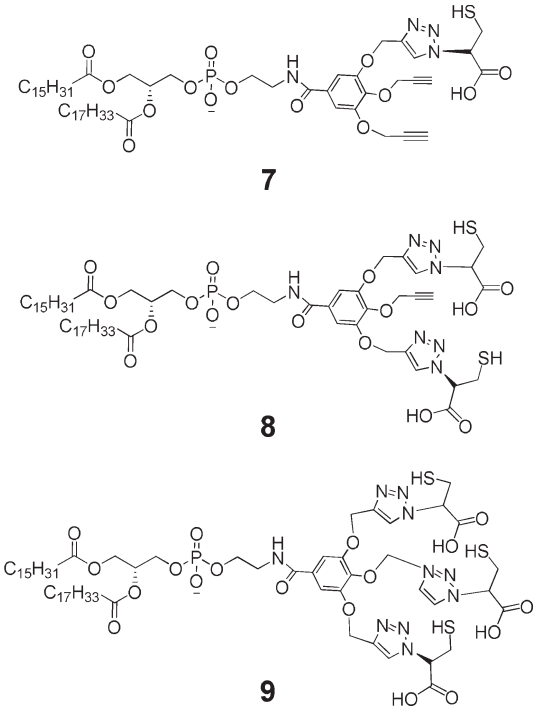


Figure 3. Percent leakage of ANTS/DPX from LUVs (a) before and (b) after polymerization. [ANTS/DPX] = 12.5/45 mM in 5 mM TES buffer. [**1**]/[POPC] = 1:2; [NaCl] = 5 M; pH = 7.0 (○), 6.0 (◊), 5.0 (□), and 4.0 (△).



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the leakage assays suggested that the low cross-linking density might not be a bad result at all—even though the CPLs were significantly more stable than the unpolymerized materials, the release started as soon as the cleavage agent was added.

The outstanding tolerance of the click reaction made it easy to incorporate cross-linkers sensitive to other stimuli. Cross-linker **5**, for example, contained a ketal bond that could hydrolyze easily under acidic conditions. Acid-triggered release is important to many delivery applications. Endocytotic delivery often exploits the higher acidity of endosomes compared to cytosols.[65] Cancerous and inflammatory tissues are also known to be more acidic than normal tissues.[66−68] We prepared CPLs with a composition of [**1**]/[POPC] = 1: 2 with a slightly modified procedure. Cross-linker **5** was not soluble in water; we thus could not add the cross-linker to preformed liposomal solution. Instead, we mixed **1**, POPC, and **5** together during lipid film formation and prepared LUVs with **5** already in the lipid bilayers. Polymerization was accomplished by Cu(I) catalysts added to the aqueous solution.

We could not use the CF leakage assay for acid-triggered release because of its acid-sensitivity.[15] Instead, we employed the

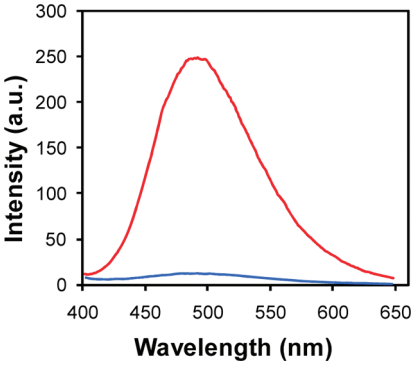

Figure 4. Fluorescence spectra of click-labeled LUVs (red) and the control LUVs (blue) after gel permeation chromatography (λ_{ex} = 345 nm). The control LUVs were prepared exactly the same as the click-labeled LUVs except that no copper catalyst was added in the process. [**1**]/[POPC] = 1:2.

ANTS/DPX assay, known to work well under acidic conditions.[69,70] ANTS is a fluorescent dye and quenched strongly by DPX. When trapped together inside the liposomes, the pair gives little fluorescence. Leakage of one or both compounds reduces the concentration-dependent quenching and can be easily detected.

The ANTS/DPX assay worked well for both unpolymerized liposomes and the CPLs. As shown by Figure 3a, leakage occurred readily when the LUVs was subjected to the 5 M NaCl osmotic stress. In general, the fastest leakage took place within the first 5 h of time and leveled off afterward. Polymerization changed the release profiles significantly. The polymerized liposomes had considerably higher stability at pH 7 (Figure 3b, ○). Whereas the unpolymerized LUVs lost over 70% of the probes after 24 h, the CPLs only lost ∼30%. As the solution became more acidic, the effect of polymerization disappeared gradually. At pH 6 (◊), for example, the 24 h leakage was ∼50% and 80% for the polymerized and unpolymerized LUVs, respectively. When the solution pH decreased further to 5 (□) and 4 (△), polymerization no longer affected the 24 h leakage, but the initial rates of release, judged by the slopes of the curves in the early stage of the assays, were clearly smaller for the polymerized liposomes.

Covalent labeling of the liposomes was extremely easy with our method. As a proof of concept, compound **6** (Scheme 1) was prepared by a one-step reaction from a commercially available probe (see Supporting Information for details). Cross-linking and fluorescent labeling occurred simultaneously when **4** and **6** were added together during polymerization. After gel permeation

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chromatography (GPC) over Sephadex G-50 resins, the CPLs emitted much more strongly than the control liposomes, in which no catalyst was added (Figure 4). GPC separates molecules by their sizes. Without cross-linking, most of the dye was removed from the control liposomes during chromatography. The weak emission from the control LUVs (Figure 4, blue) probably came from physically adsorbed **6**.

In summary, polymerization of liposomes is an effective strategy to improve their stability. Introduction of multiple "clickable" functionalities on the headgroup of the lipid and the subsequent click polymerization with cleavable cross-linkers such as **4** and **5** create novel CPLs that readily depolymerize under biologically relevant conditions. In contrast to conventional free radical polymerization, the click reaction retained the benefits of polymerization (i.e., enhanced stability) without the problem of polymer-related poor biodegradability. The simplicity of the synthesis, the facile implementation of the technique in common liposome formulations, and the convenient multivalent surface modification make this method highly attractive in the controlled release and delivery of pharmaceutical agents.

■ ASSOCIATED CONTENT

Supporting Information. Entire experimental section including synthesis and characterization of the compounds, liposome preparation, and leakage assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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